Functional studies on the shell soluble matrix of *Anodonta cygnea* (Bivalvia: Unionidae)

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ABSTRACT

The biomineralization process in molluscan shells is controlled by an extracellular organic matrix, embedded in a fluid, produced by the calcifying outer mantle epithelium (OME) and secreted within the extrapallial compartment.

In the present work, the study subject is the nacreous layer of the freshwater bivalve *Anodonta cygnea* and the functional role of its organic matrix, which is still a large field to explore. From the organic matrix it was possible to extract two fractions, but only the soluble fraction was studied. Different techniques were used, including biochemical protein characterization by electrophoresis for the extrapallial fluid and the shell, quantification and detection of the matrix proteins and glycosamynoglycans (GAGs) directly in the shell, through immunogold techniques, using SEM and ATR-IR observations. Seven protein fractions in both extrapallial fluid and shell were detected by electrophoretic analysis with molecular weights of approximately 102/106, 76/74, 66/66, 60/52, 45/43, 35/35 and 31/29 KDa, respectively. This may suggest a narrow functional correlation between specific proteins from the extrapallial fluid and the shell. Despite the low percentage of the organic matrix relative to the whole nacreous shell, it was observed that it is mainly composed of proteins (13.40–23.32 mg/ml) and GAGs (2.50–3.12 mg/ml), which appear to be very relevant on the microstructure and polymorphism organization of the major calcium carbonate fraction. In agreement, the immunogold technique showed that the shell organic matrix is mainly intercrystalline. Additionally, the common detection by infrared spectroscopy of amide groups on both soluble shell matrix and solid shell crystal fraction suggests that this molecule is one of the intracrystalline inductors of the aragonite crystals formation in the nacreous layer of *A. cygnea*.

Additional Keywords: biomineralization, extrapallial fluids, organic matrix

INTRODUCTION

The shell structure in the molluscan shell carries a good historical record that helps explain the evolution of this

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group of animals since the Cambrian period. Shells are secreted by a vast majority of the estimated 70,000–76,000 named molluscan species (estimate in Rosenberg, 2014) and their construction begins in the early stages of development and almost continuously during their entire life (Bøggild, 1930; Marin and Luquet, 2004). The shell crystals in bivalves show a great variety of morphologies and organization levels originating different microstructures and polymorphisms (Checa et al., 2007; Lopes-Lima et al., 2010). However, all of them are constituted by calcium carbonate representing 95–99% of the shell and by 1–5% corresponding to organic matrix (Duplat et al., 2006). Adult shells are highly variable being built up of one or more shell layers, each of which may have a different microstructure (Bøggild, 1930; Weiss et al. 2002). In a longitudinal section, the Unionidae shell reveals generally two calcified layers, prismatic and nacreous layers, and one outer organic layer, the periostracum, which protects the calcified layers from water dissolution (Bøggild, 1930; Moura et al. 2003; Marin and Luquet, 2004). The most common mineral polymorphs identified in the calcified layers of calcium carbonate are aragonite and/or calcite (Weiner, 1983; Checa et al., 2007). In the families Pinnidae and Pteriidae the shell has one internal layer with calcium carbonate in the aragonite form and another external in the calcite form whereas in the Unionidae family the two calcified layers are both aragonitic (Bøggild, 1930; Taylor et al., 1969; Caiping et al., 2005; Marie et al., 2007).

Although the shell calcification process occurs outside the living tissues, it is neither in contact nor directly dependent on the external environment. The process is mainly dependent on three components: a closed compartment where the calcification occurs, an ionic membrane transport and an extracellular organic matrix (Moura et al., 2003). The closed compartment filled with extrapallial fluid is bounded by the shell, the periostracum, and the calcifying outer mantle epithelium (OME). This isolation is critical to provide a supersaturated environment which is essential for the formation of crystals (Marin and Luquet, 2004). The extracellular organic matrix, secreted by the calcifying epithelium towards the extrapallial fluid, consists of a complex mixture of proteins, glycoproteins, proteoglycans and chitin (Moura, 2000; Pereira-Mouriès et al., 2002). This matrix has important roles in the physical-chemical interactions involved in crystal nucleation, polymorphic seleetion, growth, and inhibition (Marxen and Becker, 1997; Levi-Kalisman et al., 2001; Pereira-Mouriès et al., 2002).

Little is known about the extracellular organic matrix of the unionid freshwater mussel *Anodonta cygnea*. The shell exhibits the three layers already mentioned and, though the two calcified layers present different microstructures, both correspond to the same calcium carbonate polymorph, aragonite. According to Moura et al. (2000) there are 4–6 protein fractions in the calcifying fluids of *A. cygnea*.

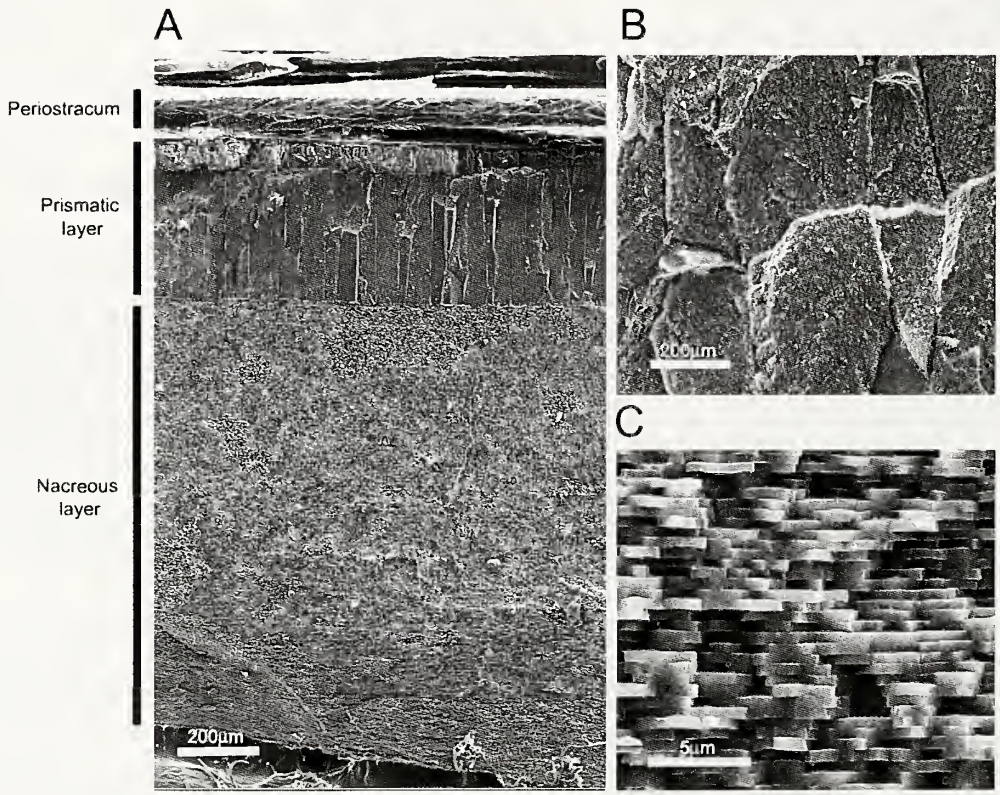

Figure 1. SEM images of the shell of *Anodonta cygnea* in back-scattered electron mode. (A) The three layers are perfectly distinct in transversal sections; magnification of prismatic (B) and nacreous (C) layers.

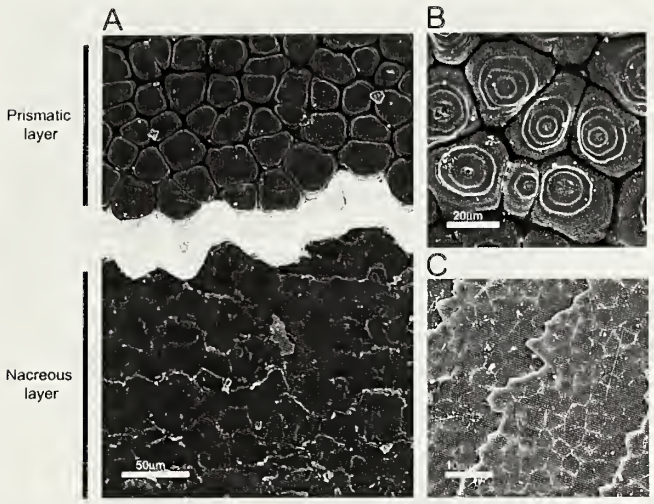

Figure 2. SEM images of the external shell surfaces of *Anodonta cygnea* in back-scattered electron mode (A). Magnification of prismatic (B) and nacreous (C) layers.

The objective of this work is to study the soluble fraction of the extracellular organic matrix from the extrapallial fluid and nacreous layer of the freshwater mussel *Anodonta cygnea,* in order to gain further knowledge about its components. The main goal was to extract and purify the organic matrix from the shell in order to quantify and analyze it by electrophoresis and Attenuated Total Reflectance-Infrared Spectroscopy (ATR-IR). The direct visualization of the organic matrix presence in the shell was also an aim of the study, through the use of immunohistochemical techniques.

MATERIALS AND METHODS

Organic Matrix Extraction, Quantification and SDS-PAGE Analysis

Freshwater bivalves, *Anodonta cygnea*, were collected from the bottom of Mira Lagoon (40°26.712N, 8°47.817W) in the end of July 2012. The nacreous layer organic matrix was extracted as described by Caiping et al. (2005) with the following exceptions: the initial amount of nacreous layer sample was higher (90 g), the dialysis was performed against ultrapure water and, at the end, the sample powder was re-dissolved in PBS buffer.

The TCA-DOC protein precipitation technique was used for the extraction of very low contents of soluble protein from the extrapallial fluid. For this, to the extrapallial fluid, was added 1/100 vol. of 2% DOC (sodium deoxycholate), followed by the addition of 1/10 of trichloroacetic acid (TCA) 100% and centrifuged for 15 min at 4 °C in a microfuge at maximum speed (15000 g). For the SDS-PAGE, the protein pellet was re-suspended in a minimal volume in PBS buffer.

In this work only the soluble fraction was analyzed. Two independent replicas of the extraction were accomplished. Total protein and glycosaminoglycans concentrations in the shell were determined respectively according to the methods of Bradford (1979) and Whiteman (1973). Protein fractions from the extrapallial fluid and shell samples and molecular weight standard were separated and analyzed using a mini-SDS-PAGE system in 8% polyacrylamide gels at 130V during 60 min and stained with silver nitrate (Gromova and Celis, 2006).

Peptide Mass Mapping from the Extrapallial Fluid and Shell Protein Band

The observed protein band from the SDS-PAGE gel was cut and transferred to the Eppendorf tubes before being sent to Alphalyse, Inc. (USA) for peptide mass mapping. From the seven protein bands detected, only the five marked bands with highest expression were sent for analysis (Figure 4). The protein samples were reduced and alkylated with iodoacetamide, i.e., carbamidomethylated, and subsequently digested with trypsin and chymotrypsin. Trypsin cleaves after lysine and arginine residues. The resulting peptides were spotted directly onto an anchorchip target or were concentrated on a C18 ZipTip micropurification column and eluted onto an anchorchip target for analysis on a Bruker Autoflex Speed MALDI TOF/TOF instrument. The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination (MALDI-MS).

Polyclonal Antibodies Production and Visualization of the Shell Organic Matrix by Immunogold Technique

The polyclonal antibodies were produced in two rabbits. For each immunization 100 µg of organic matrix were injected in the intradermic neck region. The immunization procedures were reinforced after 30 and 42 days. Bleedings were carried at 0 (pre-immune serum), 30, 42, and 54 days. The sera were then titrated by standard ELISA assays, for evaluation of the more appropriate antibody concentration. The third bleeding was then chosen and used in the following procedures. IgGs were purified in a protein G column (GE Healthcare) and used in the immunogold assays. This assay was performed as described by Marin et al. (2007). Briefly, the

Table 1 Quantitative results of organic matrix extraction from the nacreous layer of *A. cygnea*.

Sample	Initial shell (g)	Final organic matrix powder (g)	Organic matrix % (w/w)	Total proteins (mg/ml)	Total GAGs (mg/ml)
1	90	0,0347	0,039	23,32 ± 0,27	3,12 ± 0,11
2	90	0,0476	0,053	13,40 ± 0,35	2,05 ± 0,03

nacreous layer was broken in small pieces and etched with EDTA 1% (w/v), pH 7.5 during 2-3 min with agitation. The pieces were then incubated overnight with a 1:3000 dilution of the IgGs produced against the organic matrix. The secondary antibody used (Anti-rabbit IgG – Gold antibody produced in goat, affinity isolated antibody, aqueous glycerol suspension, 5 nm, Sigma) was diluted 1:400 and incubated during 2 h. The silver enhancement was performed with a Silver Enhancer Kit by Sigma. As a negative control the pieces were first incubated with pre-immune serum. The results were observed through scanning electron microscopy (SEM).

Scanning Electron Microscopy Imaging (SEM) of the Shell and Attenuated Total Reflectance-Infrared Spectroscopy (ATR-IR) of Aqueous Shell Matrix.

Untreated shell pieces were gold-coated (FINE-COAT Ion sputter JFC-1100) and glued to aluminum stubs for SEM observations using JEOL JSM-35C scanning electron microscope operated at 15 keV in Centro de Materiais da Universidade do Porto (CEMUP). Shell pieces treated with immunogold technique were carbon-coated and analyzed in back-scattered electron mode at 15 keV.

Two aqueous samples of soluble organic matrix were analyzed by ATR-IR using a Bruker Tensor-27 spectrometer equipped with a DTGS (deuterated triglycine sulfate) single detector plate and a horizontal ATR unit, where a horizontal ZnSe ATR crystal was mounted at 45° in a 30 ml rectangular cell made of polypropylene. Samples were run in the frequency range 800–4000 cm^{-1}. Additionally, few milligrams of nacreous shell layer were extracted from the freshwater mussel *A. cygnea* and then were analyzed by infrared spectroscopy in absorbance mode using a BRUKER Tensor-45 spectrometer. The pellet disks of 1.5 cm diameter were prepared by mixing 1 mg of sample with 200 mg KBr and pressing at 10 Kg/cm^2.

RESULTS AND DISCUSSION

Characterization of the Natural Microstructure of *Anodonta cygnea*

SEM observations (Figures 1 and 2) highlight the nature of the shell microstructure in the freshwater mussel *A. cygnea*. The three different layers are evident: one organic (periostracum) and two calcareous layers (prismatic and nacreous). In the prismatic layer the aragonitic crystals are organized in prisms covered with organic matrix surrounding them, whereas in the nacreous layer the crystals are organized with the shape of tablets with organic matrix between them.

In this work, we have mainly focused on the nacreous layer, specifically in its organic matrix. Regarding the shell nacreous layer, the quantitative results of its main organic components are presented in Table 1. As described in the literature for *A. cygnea* by Moura et al.

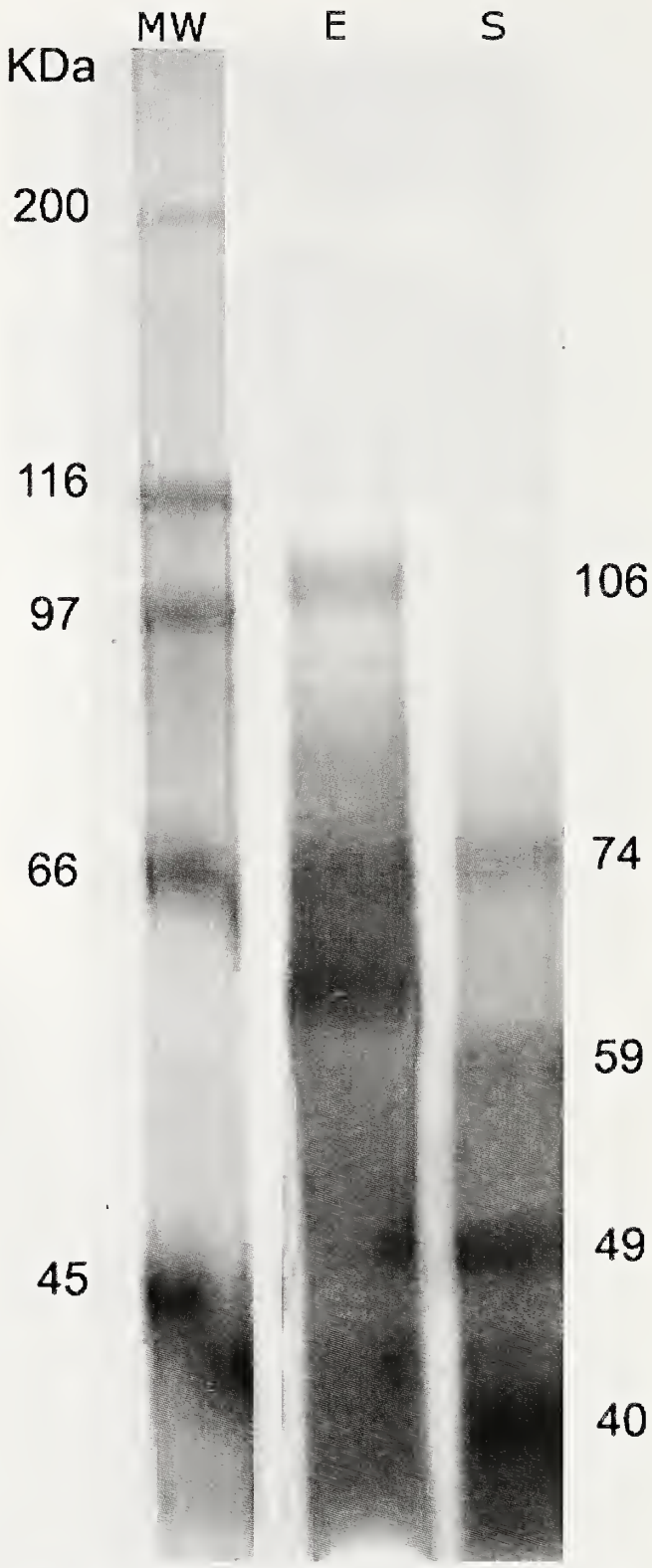

Figure 3. SDS-PAGE of the soluble organic matrix extracted from the extrapallial fluid (E) and shell nacreous layer (S) of *Anodonta cygnea*. MW: molecular weight standards; E: Protein fraction from the extrapallial fluid; S: Protein fraction from the shell.

(2000) and for other species of bivalves (Marie et al., 2007), it was also stated that the organic matrix represents a small amount of the total shell weight. The majority of this matrix is represented by proteins (13.40–23.32 $mg.ml^{-1}$), while the glycosaminoglycans (GAGs) were found in lesser amounts (2.50–3.12 $mg.ml^{-1}$). Although present in smaller amounts, GAGs are always found in the organic matrix, denoting their importance in most biomineralizing systems (Pereira-Mouriès et al., 2002; Moura et al., 2000, 2003; Lopes-Lima et al., 2005, 2010). Naturally, the total protein and GAGs contents were higher in the shell matrix compared to the organic fluids as reported by Moura et al. (2000) for the same period. In fact, these results confirm that the shell matrix structure act as a sponge. Furthermore, while results of Moura et al. (2000) reported 6 protein fractions on the haemolymph and extrapallial fluids along the year, the present study adds complementary data based on the detection of seven different protein bands in extrapallial fluid which are similar to others in the shell matrix of *A. cygnea* (Figure 3).

The SDS-PAGE technique recorded seven protein fractions with molecular weights bands of approximately 102-106, 76-74, 66-66, 60-52, 45-43, 35-35, and 31-29 KDa,

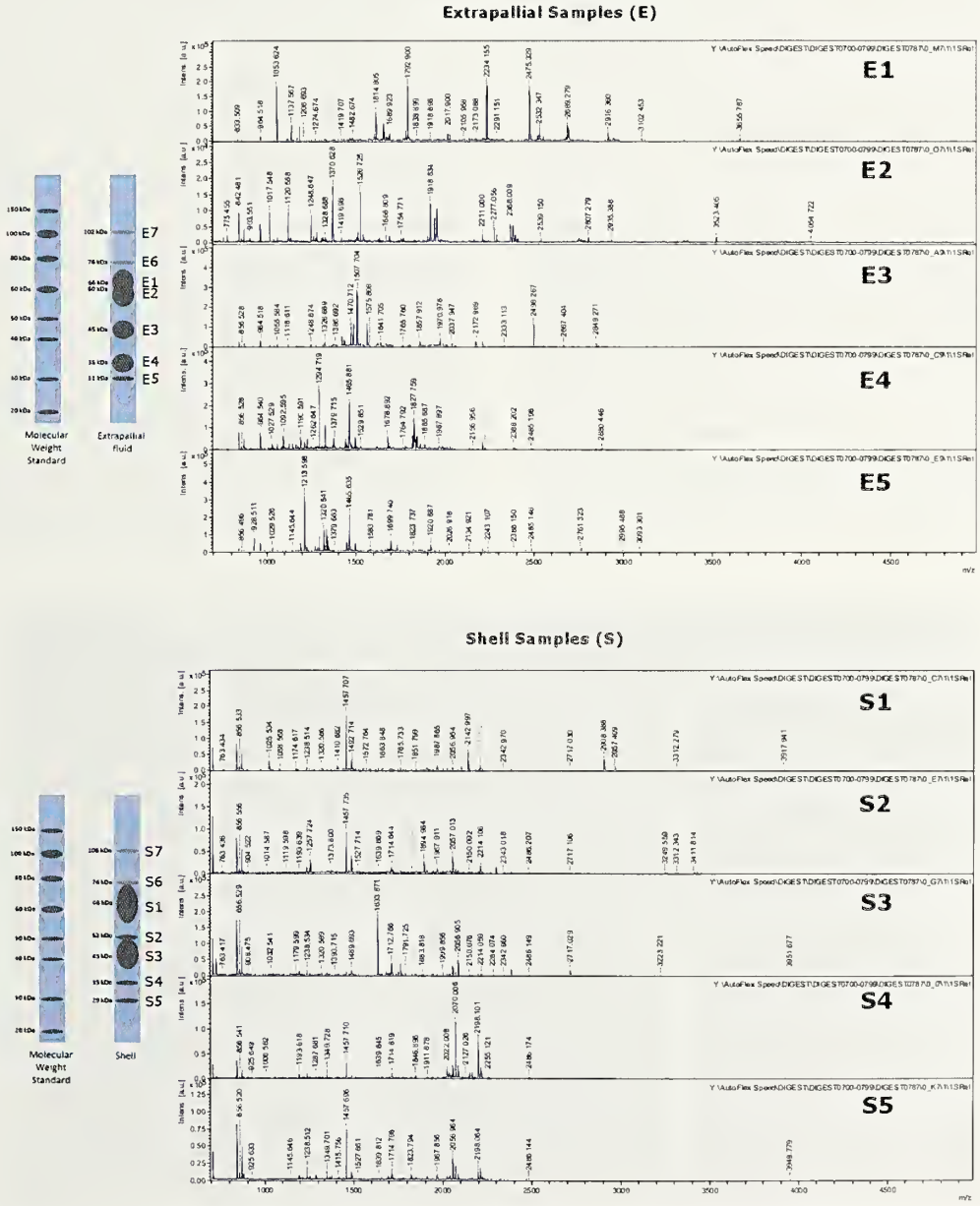

Figure 4. Diagram of a spectra of peptides mass mapping of soluble organic matrix extracted from the extrapallial fluid (E1-E5) and shell nacreous layer (S1-S5) of *A. cygnea* by MALDI-MS determination.

respectively in the extrapallial fluids and in the shell matrix (Figure 3). However, from these seven bands, only five were submitted for peptide mass analysis, since the two heaviest (74-106 KDa) showed poor resolution. According to Moura et al. (2000), the protein bands in the fluids can show different expression levels along the year, which can be correlated with its own functional role in the shell. Similar studies by electrophoresis have been previously performed in at least three families of mollusks, two of them in the Bivalvia, and the number of protein fractions found was also low (Marxen and Becker, 1997; Pereira-Mouriès et al., 2002; Caiping et al., 2005; Marie et al., 2007). Yet, regarding the number of bands, these results revealed great conformity with previous studies, which state between 4 to 6 bands, depending on the mussel species (Misogianes and Chasteen, 1979; Keith et al., 1993; Moura et al., 2000). Curiously, the new data showed that the seven protein fractions present close molecular weight both on the shell matrix and extrapallial fluid samples. This finding may point out that specific proteins in the extrapallial fluid are involved on the shell biomineralization process in *A. cygnea*. Additionally, the MALDI-MS analysis revealed a large number of peptides per protein band in all extrapallial and shell fractions. There are similarities in the peptide mass from equivalent protein bands among different extrapallial samples and the same occurs in the shell samples. Furthermore, similarities were also found when comparing equivalent fractions from fluid and shell samples (Figure 4). These statements may eventually predict the presence of similar proteins in fluid and shell samples. All these aspects, in general, lead us to propose a specific functional role of fluid proteins on the shell formation.

The results of the immunogold assay in *A. cygnea* to detect organic matrix protein proved to be effective and useful. In the back-scattered electron mode, the gold particles (covalently bound to the secondary antibody) appeared as tiny bright spots. As shown in Figure 5, these tiny bright spots were mainly found in the spaces between the calcareous crystals, though some were also observed within the crystals, denoting the presence of organic matrix as a fundamental component on the biomineral phase. Actually, these spots were distributed either around the columnar structure of aragonite crystals or filling the spaces between nacreous layers of aragonite crystals. This confirms that the organic matrix may play an essential role on the mineral formation and organization in both vertical and horizontal axes in bivalve shell (Krampitz et al., 1983; Checa, 2000).

Molecular Vibrations of the Organic Matrix of the Nacreous Layer

Infrared spectroscopy is a resource for a characterization at a molecular level of the structure and bonding of surface functional groups and adsorbed species. In this study, ATR-IR spectra of aqueous organic matrix shows two molecular vibrations at 3300 cm^{-1} and 1640 cm^{-1} (Figure 6) corresponding to amide group (amide-A). The band observed at 3300 cm^{-1} is very broad because the O-H stretching band appears as a typical polymeric hydrogen bonded envelope near 3300 cm^{-1}. This means that the polymeric hydrogen bonding donors are part of the amide-A (C-H) group which displays strong and broad C-H asymmetric stretching absorptions at 3300 cm^{-1}. The molecular vibration at 1640 cm^{-1}

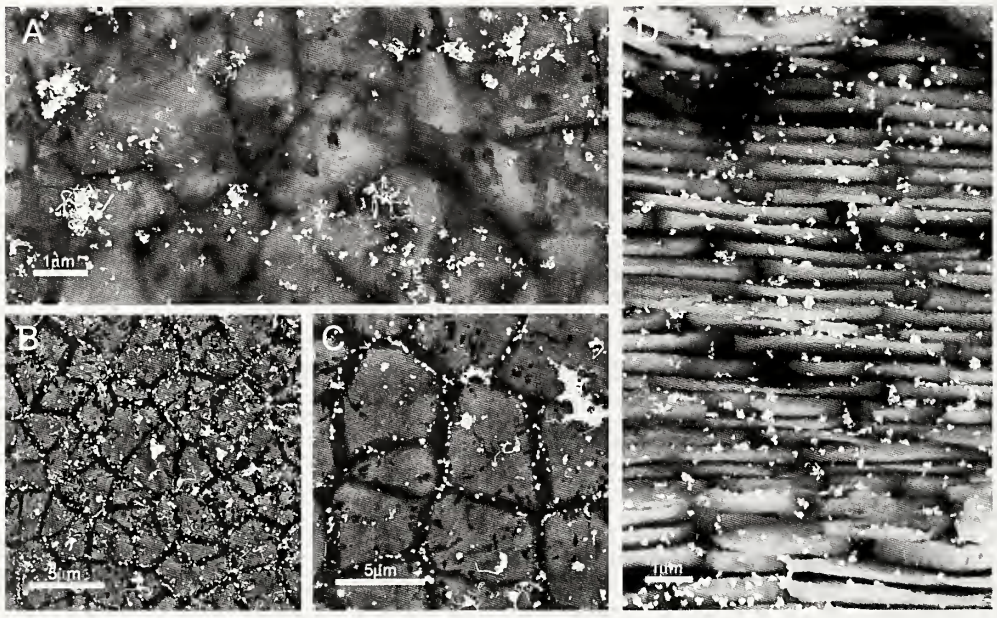

Figure 5. Immunogold assay results. Topographical view of (A) negative control, (B) and (C) test with positive results; (D) positive result in a transversal view.

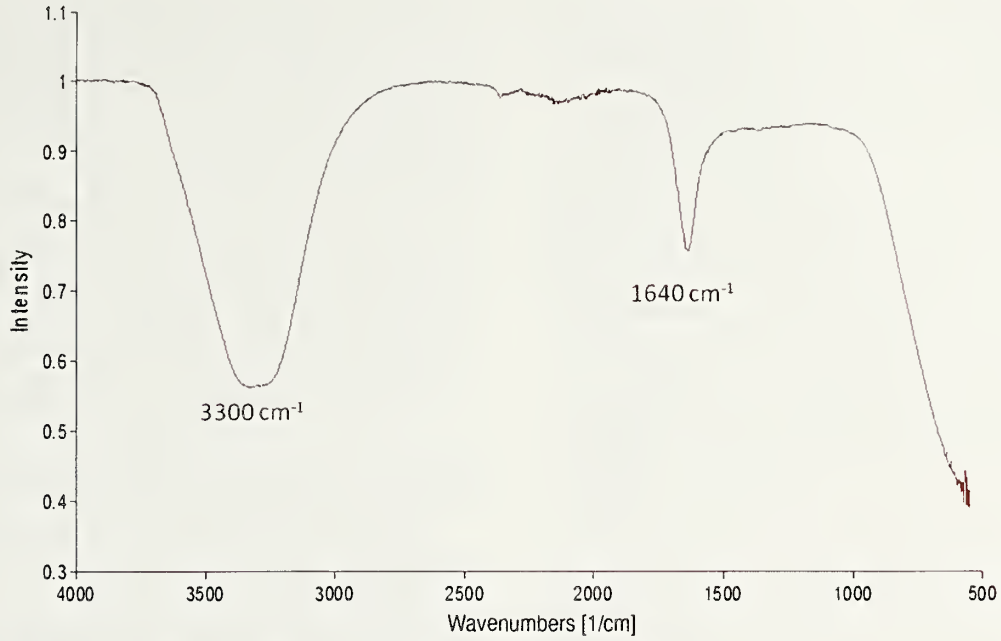

Figure 6. ATR-IR spectra of aqueous organic matrix from nacreous layer *A. cygnea*.

represents a symmetric C-O stretching. A band may be attributed to the presence of some C-O (amide 1°-band).

On the other hand, nacreous solid samples analysed (Figure 7) show a strong stretching vibration of CO_3^{2-} at 1470 cm^{-1} corresponding to ν_3 vibration mode, which is relevant for aragonite structure. The ν_2 asymmetric bending vibration at 860 cm^{-1} is also observed, which suggests a higher Ca^{2+} contribution than Sr^{2+} or Mg^{2+} in aragonite structure. The vibration at 710 cm^{-1} also corresponds to the ν_4 vibration mode (O-C-O) in plane bending of CO_3^{2-}. The vibration band at 1016 cm^{-1} corresponds to the ν_1 vibration mode of CO_3^{2-}. The shoulder at about 1600 cm^{-1} is related with amide-I functional group.

CONCLUSIONS

In general, this study provides new information on the organic shell matrix of *Anodonta cygnea* and confirmed similarities with other studied families. Despite its small

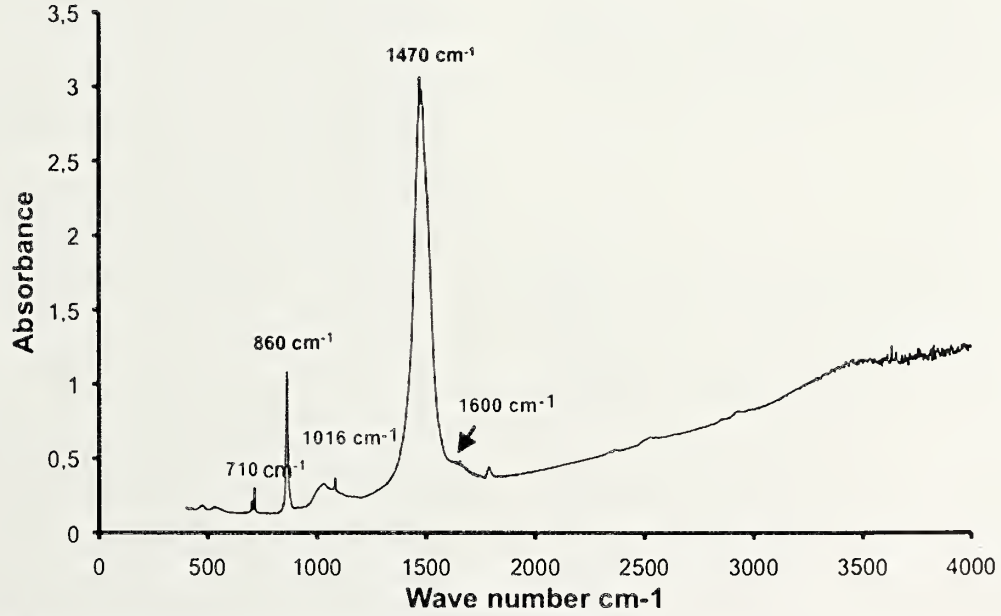

Figure 7. FT-IR spectrum of the internal nacreous layer (with aragonitic structure) collected from the freshwater mussel *Anodonta cygnea* shell.

percentage, the organic matrix, constituted mainly by proteins and sulfated GAGs, has a relevant influence on microstructure (proteins) and on the crystal nucleation (sulfated GAGs) during the formation of the calcium carbonate layers (Moura et al., 2000; Lopes-Lima et al. 2005). Additionally, it has been suggested (Tong et al., 2002) that while the intra-crystalline organic matrix, mainly composed of small negatively charged molecules, provides nucleating points and induces nucleation process (Nudelman et al., 2006), the inter-crystalline organic framework possesses a more complex organic composition and is responsible for supporting, limiting size and shape, and determining crystal growth spatial orientation.

In the present study, fluid and nacreous protein fractions, composed by similar weight molecular peptides as found by electrophoretic and MALDI-MS analysis as well as the protein matrix observed in the nacreous by SEM hystochemical techniques, seem to play mainly an inter-crystalline role in the shell biomineralization. On the other hand, the results obtained from soluble matrix and solid nacreous samples by infrared spectroscopy analyses, showing the similar occurrence of an amide group, probably points out an intra-crystalline factor contributing for the aragonite crystal formation in the nacreous layer of *A. cygnea*. In fact, according to Choi and Kim (2000), Xiao et al. (2005) and Kasat et al. (2006), the great electronegativity of oxygen allows amides to act as H-bond acceptors changing the H-bonding states of C=O groups and consequently may define secondary structure and polymer crystallinity. Possibly, the amide-I group is involved in the calcium carbonate intra-crystalline structure acting as an inductor of aragonite polymorph.

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